Expanding the Toolkit for the Serine Hydrolases

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In this issue of *Chemistry & Biology*, Cognetta et al. (2015) describe new pharmacological tools, including *N*-hydroxyhydantoin-containing carbamate inhibitors and an activity-based probe, for palmitoyl protein thioesterase 1 and alpha, beta-hydrolase domain-4 that expand the toolkit for the serine hydrolases.

Serine hydrolases are involved in a number of physiological and pathophysiological processes, including neurotransmission, digestion, immune response, and the clotting cascade (Bachovchin and Cravatt, 2012). The enzymes catalyze the hydrolysis of esters, amides, or thioesters using a base-activated serine nucleophile. There are roughly 240 predicted serine hydrolases in the mammalian proteome, with one-half characterized as metabolic serine hydrolases and the other half as serine proteases/peptidases (Bachovchin and Cravatt, 2012). Metabolic serine hydrolases hydrolyze endogenous small molecules and some of the substrates and their cognate enzymes are well characterized; for example, the neurotransmitter acetylcholine is cleaved (and inactivated) by acetylcholinesterase (ACHE); the endocannabinoids 2-arachidonoylglycerol and anandamide are hydrolyzed by monoacylglycerol lipase and fatty acid amide hydrolase, respectively; and neutral lipids, such as triacylglycerols and cholesteryl esters, are hydrolyzed by adipose triglyceride lipase and hormone-sensitive lipase. However, the physiological functions of most mammalian serine hydrolases remain poorly characterized.

The majority of serine hydrolases in mammals still lack selective inhibitors for their functional characterization in terms of their endogenous substrates and cellular functions. Absence of effective small-molecule probes or pharmacological tools for the bulk of this enzyme class limits the systematic analysis of protein function. To address this gap, activity-based protein profiling (ABPP) enables the activities of enzymes with conserved catalytic mechanisms, such as the serine hydrolases, to be evaluated in their native environments within tissues and cells (Niphakis and Cravatt, 2014). This chemoproteomic strategy has been extremely useful for identifying selective and potent small molecule inhibitors of several members of the serine hydrolase superfamily, including those that are uncharacterized in terms of their substrates and products. The most potent serine hydrolase inhibitors are those that covalently modify and irreversibly inactivate the catalytic serine nucleophile. Of the various small molecules that chemically react with the serine residue, the carbamates have proven to be a versatile chemotype for inhibiting the activity of serine hydrolases. The carbamate chemotype has been used for a long time as an electrophile that can complement the nucleophilic serine residue in serine hydrolases, resulting in the formation of a covalent bond between the electrophilic carbonyl carbon and the serine oxygen nucleophile (Figure 1A). For example, N-methylcarbamates have been used as pesticides since the 1950s and exert their toxicity by acting as a slowly turned-over ACHE substrate, effectively leading to the irreversible inactivation of ACHE and the buildup of the neurotransmitter acetylcholine in the synaptic cleft (Casida and Durkin, 2013). More recently, the carbamate chemotype has been embedded into various chemical scaffolds and developed as chemical probes and pharmaceuticals to treat disease (Bachovchin and Cravatt, 2012). Depending on the structure of the staying group, the covalent carbamylated-enzyme adduct that is produced is essentially irreversible and enzyme function is inactivated (Crow et al., 2012).

In this issue, Cognetta et al. (2015) describe a library of *N*-hydroxyhydantoin (NHH)-containing carbamate inhibitors that evolved from related *N*-hydroxysuccinimide (NHS)-containing carbamates (Chang et al., 2013). As noted by the authors, the substrate selectivity of enzymes is predictive of features important for inhibitor binding, and the catalytic efficiencies of serine hydrolases are strongly influenced by the leaving group structures of substrates. Therefore, the objective of their study was to increase the number of carbamate inhibitors by diversifying the structures of its leaving group. The rationale was that these new compounds would have the potential to engage previously untargeted serine hydrolases in proteomes and provide leads for further structural optimization. The NHH-containing carbamate was chosen as a scaffold over *N*-hydroxysuccinimide because of the greater potential for structural diversification of the NHH-containing leaving group (Figure 1B). Further modifications were made to both the staying (carbamylating) and leaving (NHH) groups to optimize potency and selectivity. Structural refinement of the lead compounds provided a group of compounds that selectively inhibited a number of serine hydrolases whose activities had previously not been inhibited by small molecules. These included alpha, beta-hydrolase domain-4 (ABHD4) and palmitoyl protein thioesterase 1 (PPT1). ABHD4 hydrolyzes N-acyl phospholipids (Lee et al., 2015), whereas PPT1 removes fatty acyl groups from cysteine residues in proteins (Lu and Hofmann, 2006). The biological function of these hydrolases in vivo, however, remains enigmatic. A potent and selective inhibitor of PPT1, designated ABC44, was identified with an IC_{50} of 0.1 μM (in situ, ABPP). Remarkably, of the 44 serine

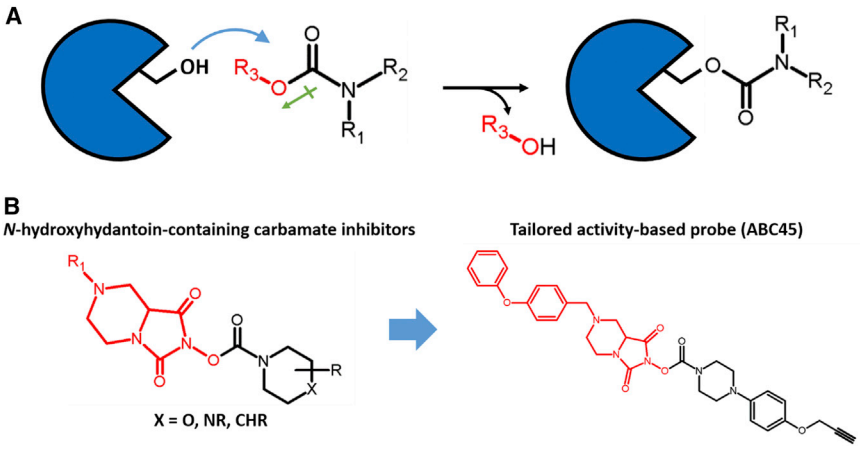

Figure 1. Development of *N*-Hydroxyhydantoin-Containing Carbamate Inhibitors and a Tailored Activity-Based ABC45 that Enable the Study of Previously Difficult to Analyze Serine Hydrolases Such as PPT1

(A) The base-activated serine nucleophile of the serine hydrolase is covalently modified by the electrophilic carbamate chemotype (leaving group is shown in red; the carbamylating group is shown in black).

(B) Cognetta et al. (2015) describe new inhibitors in which the *N*-hydroxyhydantoin-containing leaving group (shown in red) was synthetically modified to afford new inhibitors that selectively inactivate palmitoyl protein thioesterase 1 (PPT1) and ABHD4. This scaffold was used to generate an alkyne-tagged activity-based chemoproteomic probe (termed ABC45) that reacts directly with PPT1 and ABHD4 in complex proteomes.

hydrolase activities measured in the human PC3 cell line by ABPP-SILAC (stable isotope-labeling by amino acids in cell culture) following in situ treatment with ABC44 (0.1 μM, 4 hr), the only detectable off-targets were ABHD6 and CPVL. Development of selective NHH carbamate inhibitors with restricted reactivity not only provided leads for drug development but also a novel activity-based probe to characterize previously difficult to analyze serine hydrolases. The synthesis of a tailored activity-based probe (ABC45; Figure 1B), which is an alkyne-containing clickable probe, provides a tool that can directly read out the activity state of PPT1 and ABHD4 in native biological systems. This is an important contribution to the chemoproteomic toolkit, because the broad-based serine hydrolase activity probe fluorophosphonate-rhodamine (or biotin) does not react efficiently with these particular enzymes. Our own work had previously shown this for PPT1 (Wang et al., 2013). The synthesis of the tailored activity-based probe ABC45 with the clickable alkyne tag will be useful for studying PPT1 in intact cells and animal models. It will also permit the development of more potent and selective inhibitors that will enable its function to be perturbed in different physiological contexts. Mutations in PPT1 that inactivate enzyme activity cause a rare neurological disease termed infantile neuronal ceroid lipofuscinosis (INCL), but the biochemical basis for the pathology is unclear. In addition to new chemical tools applied to PPT1, a first-generation inhibitor of ABHD4 was also developed that showed good selectivity. ABHD4 in cells and tissues was also directly labeled by the new activity-based probe ABC45, which will help to guide future studies that optimize the potency and selectivity of the ABHD4 inhibitors.

Thus, the study by Cognetta et al. (2015) provides new pharmacological tools that target serine hydrolases PPT1 and ABHD4 with good selectivity in vivo. These enzymes had heretofore been difficult to study because of their lack of reactivity toward broad-based fluorophosphonate activity-based probes. The new inhibitors and tailored activity-based probes will be useful as chemical probes to investigate the function of these enzymes in cell and animal models and will also serve as leads for drug development. The findings of the paper add to the remarkable list of chemical tools that have been developed for the serine hydrolase class. These have been vitally important contributions to the research community.

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